American Journal of Medical Genetics 63:167–174 (1996)

Tracing the Pathway Between Mutation and Phenotype in Osteogenesis Imperfecta: Isolation of Mineralization-Specific Genes

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The brittleness of bone in people with lethal (type II) osteogenesis imperfecta, a heritable disorder caused by mutations in the type I collagen genes, arises from the deposition of abnormal collagen in the bone matrix. The inability of the abnormal collagen to participate in mineralization may be caused by its failure to interact with other bone proteins. Here, we have designed a strategy to isolate the genes important for mineralization of collagen during bone formation. Cells isolated from 16-day embryonic chick calvaria and seeded post-confluence in culture deposited a mineralized matrix over a period of 2 weeks. Chick skin fibroblasts seeded and cultured under the same conditions did not mineralize. Using RT-PCR, we prepared short cDNAs (~300 bp) corresponding to the 3′ ends of mRNA from fibroblasts and separately from the mineralizing calvarial cells. Subtractive cDNA hybridization generated a pool of cDNAs that were specific to mineralizing calvarial cells but not to fibroblasts. Screening of 100,000 plaques of a chick bone ZAP Express cDNA library with this pool of mineralizing-specific cDNAs identified ten clones which comprised full-length cDNAs for the bone proteins osteopontin (eight of the ten positives), bone sialoprotein II (one of the ten positives), and cystatin (one of the ten positives). cDNAs for type I collagen, fibronectin, alkaline phosphatase, house-keeping genes, and other genes expressed in fibroblasts were not identified in this preliminary screen. The pool of short cDNAs is likely to comprise cDNAs for further bone-specific genes and will be used to screen the entire bone cDNA library of 4.2 million clones. © 1996 Wiley-Liss, Inc.

KEY WORDS: bone, collagen, osteogenesis imperfecta, genes, subtraction, RNA

INTRODUCTION

Osteogenesis imperfecta (OI), a heterogeneous group of disorders characterized by brittleness of bone, is caused by mutations in the COL1A1 and COL1A2 genes that respectively encode the two proα1(I) and one proα2(I) chains of the type I procollagen trimer [for review see Byers and Steiner, 1992]. Type I procollagen is processed to type I collagen by the specific removal of the amino- and carboxyl-terminal domains and the collagen self-assembles into fibrils in the extracellular matrix. The chains comprising the triple helical domain of type I collagen are each constructed from repeating Gly-X-Y triplets, in which X and Y can be any amino or imino acid (except for tryptophan or cysteine). Glycine, with its small side chain, is essential at every third residue position for triple helix formation. Over 100 mutations including single base pair changes, deletions, insertions, premature stop codons, and splicing mutations within the COL1A1 and COL1A2 genes have been described to cause forms of OI that range in phenotype from mild to lethal [Kadler, 1995]. The most frequent mutations are single base pair substitutions in either one of the two alleles for COL1A1 or COL1A2 that alter a codon for glycine in the triple helical domain of the chain to that of another amino acid. These mutations decrease the thermal stability of the triple helical molecules, delay the rate of folding of the precursor procollagen at physiological temperatures, increase the level of posttranslational modification of the chains, impair the rate of export from the cell of those molecules containing mutant chains [for review, see Byers and Steiner, 1992], and can delay the rate of cleavage of the procollagen molecule to collagen by the

Received for publication January 11, 1996.

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Dedicated to Jürgen W. Spranger on the occasion of his 65th birthday with admiration and best wishes.

enzyme procollagen N-proteinase [for review, see Lightfoot et al., 1994]. Experiments in vitro have suggested that abnormal collagen molecules containing mutated α-chains are more slowly incorporated into fibrils than normal collagen molecules [Torre Blanco et al., 1992; Kadler et al., 1991].

Despite knowledge of the molecular genetic basis of OI and the effect of mutations on the structure and stability of the type I procollagen molecule, little is known about the precise mechanisms whereby these mutations cause brittle bones. To gain further insight into the pathway between mutation and phenotype in OI, we have previously examined the collagen fibrils isolated from bone of two infants with lethal (type II) OI [Culbert et al., 1995]. The individuals were heterozygous for single base pair substitutions in COL1A1 that changed the codon for glycine 220 in one individual to aspartic acid and the codon for glycine 664 to arginine in the other individual. In normal bone, collagen fibrils are a template for mineralization in which crystallites of hydroxyapatite become incorporated into the fibril [Traub et al., 1989]. Our examination of normal bone showed that about 70% (by number) of the fibrils in bone were encrusted with plate-like crystallites of hydroxyapatite. In contrast, when age- and site-matched bone was examined from the infants with OI, only about 5% (by number) of the collagen fibrils contained crystallites and the crystallites were sometimes poorly aligned with the long axis of the fibrils. Biochemical analyses showed that OI bone contained both normal and abnormal collagen molecules. These findings suggested to us that the presence of abnormal collagen in the fibrils prevented the normal collagen in the same fibril from providing an adequate template for incorporation of hydroxyapatite crystallites.

Precisely how abnormal collagen prevents mineralization is not understood. It is known that normal collagen fibrils formed in vitro from purified solutions can provide a template for the formation of crystallites from super-saturated solution of calcium and phosphate. However, the process is slower and less extensive than that occurring in vivo [Endo and Glimcher, 1989; Grynpas et al., 1989]. This observation supports the notion that additional factors present in vivo may bind the surface of fibrils and thereby accelerate the seeding of mineral onto fibrils. It is possible, therefore, that mineralization-impaired fibrils in bones of people with OI lack the ability to bind such factors.

As a prelude to testing these hypotheses we set out to identify those factors that are crucial for mineralization. For this purpose, we established a chick calvarial cell culture system which synthesized mineralized collagen fibrils [Gerstenfeld et al., 1987] and a chick fibroblast culture which synthesized non-mineralized collagen fibrils. We then used a reverse transcription polymerase chain reaction (RT-PCR) based cDNA subtractive hybridization technique [Brady and Iscove, 1993; Brady et al., 1995] to isolate genes expressed in the calvarial culture but not in the fibroblast culture. The subtracted cDNA probe obtained by this procedure was used to screen a chick cDNA library which contained genes involved in the mineralization of chick calvaria. In a limited screen of the cDNA library we isolated the genes for osteopontin [Mark et al., 1987], bone sialoprotein-II [Bianco et al., 1991], and cystatin [Isemura et al., 1986]. We now plan to use our subtracted cDNA probe to conduct a more extensive search for further genes that may have important roles in the mineralization process.

MATERIALS AND METHODS

Chick Calvarial Cell Culture

Cells were established from 16-day-old embryonic chicks as described previously [Gerstenfeld et al., 1987]. Cultures were plated at low density ($6.6 \times 10^3/cm^2$) and incubated for 3 weeks in first passage with media changes every 3 days. Cells were subcultured and replated at 2.0×10^4 cells in 10% FCS in BGJ_b (Fitton-Jackson modification) medium [Gerstenfeld et al., 1987]. After 2 days cells were refed with BGJ_b supplemented with 25 μg/ml ascorbate and 10 mM β-glycerophosphate. Cells were stained for alkaline phosphatase using the Sigma Fast™ kit and for mineral using the von Kossa stain [Clark, 1981] at day 9 and day 15 of secondary culture.

Chick Tendon Fibroblasts Culture

Tendons were removed from 16 day embryonic chicks and washed in PBS. Matrix-free cells were generated by treatment with bacterial collagenase as previously described [Dehm and Prockop, 1972] and the cells were plated at the same density and cultured under the same conditions as secondary cultures of calvarial cells [Gerstenfeld et al., 1987].

cDNA Library Manufacture

Both chick calvarial cells in culture and chick calvaria tissue were used as sources of RNA for cDNA library construction. mRNA was extracted using mRNA isolation kits (Stratagene) from 10^8 chick calvarial cells in culture (grown to 15 days of secondary culture) and 3 g of calvarial tissue dissected from 14 day chick embryos. A ZAP express™ cDNA library was commercially prepared by Stratagene from an equal amount of each of the two samples of mRNA. cDNAs were cloned into the *Eco*RI restriction site of the multiple cloning region of the lambda vector. The library contained 4.2×10^6 pfu. The ZAP express vector was chosen since inserts can be excised out of the phage in the form of the kanamycin resistant pBK-CMV phagemid using filamentous helper phage [Short and Sorge, 1992; Short et al., 1988] thus eliminating the need for subcloning.

Subtractive Hybridization

Total RNA was extracted from cultured chick fibroblasts and from day 9 and day 15 chick calvarial cells using the acid-phenol method [Chomczynski and Sacchi, 1987]. The recently described method of poly(A) PCR [Brady and Iscove, 1993] was used to generate representative cDNA pools from the fibroblast (driver) and calvarial (tracer) RNA. First strand cDNA synthesis was carried out using a dT_{24} oligonucleotide primer and 1 μg of total RNA. The reverse transcription incubation time was for 15 min to ensure that all transcripts were between 100 and 500 base pairs. A poly(dA) tail was added to the single stranded cDNA

using terminal transferase and dATP as previously described [Brady and Iscove, 1993]. The tailed cDNAs were amplified in a primary PCR with the dT_{24} primer. This primary PCR was performed in a total of 50 μl containing 500 pmoles of primer in a reaction buffer of 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 1.25 mM of each dNTP, and 2.5 units of Taq polymerase. PCR was performed for 40 cycles of 94°C for 1 min, 42°C for 1 min, and 72°C for 1.5 min. Reamplification of the primary PCR products was performed with a single primer that differed for the driver and tracer. The primer used to amplify the driver was: NotI-dT (5′ CAT TCG AGC GGC CGC T_{24} 3′). The primer used to amplify the tracer was: Kvd-dT (5′ GGT AAC TAA TAC GAC TC 3′). PCR reactions were performed in a total of 100 μl containing 300 pmoles of primer, 1 μl of the primary PCR in the same reaction buffer as above plus 1 mM of each dNTP and 2.5 units of Taq polymerase. PCR was performed for 30 cycles of 94°C for 1 min, 42°C for 1 min, and 72°C for 1 min. Subtractive hybridization was performed by hybridizing, in a ratio of 20:1, denatured, photobiotinylated driver cDNAs, and non-photobiotinylated tracer cDNAs followed by extraction of the photobiotinylated material with phenol/streptavidin as previously described [Brady et al., 1995]. Four successive rounds of subtraction (S1 to S4) were performed using both day 9 and day 15 chick calvarial poly(A) cDNA as tracer versus the chick poly(A) cDNA as driver. Re-amplification by PCR of the product remaining after each subtraction was performed using the Kvd primer and the conditions described above.

Labelling of cDNA Probes

cDNA pools and cDNA probes were labeled with Klenow and ^{32}P(dCTP) using the Pharmacia oligo labeling kit. Labeled cDNA was separated from unincorporated nucleotides on Sephadex G50 spun columns [Sambrook et al., 1989].

Production of a 3′ Chick COL1A1 cDNA Probe

A cDNA probe to the most 3′ 351 base pairs of the chick COL1A1 gene was prepared by RT-PCR of RNA extracted from calvarial cells. The primers for the RT-PCR reaction were designed to hybridize to the 3′ cDNA end of COL1A1. The sequence of the sense primer was 5′ AAC CCG GCT GAT GTC GCC ATC CAA C 3′ and for the antisense primer 5′ GGG CCG ATG TCA ATG CCA AAT TC 3′. The PCR program consisted of incubations at 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min for 30 cycles.

Southern Blot Preparation and Hybridization

Approximately 100 ng of tracer and driver and reamplified products of the subtractions were separated on 1.4% agarose gels and blotted onto nitrocellulose using standard methods [Sambrook et al., 1989]. The blots were prehybridized, hybridized to ^{32}P-labeled probes and washed at 65°C using the Church hybridization system [Church and Gilbert, 1984].

Screening of the Chick Calvarial cDNA Library

The protocols, cell strains and phage stocks required for the use of the Stratagene ZAP Express™ EcoRI chick calvarial cDNA library were provided by Stratagene. Approximately 100,000 pfu were screened using the S4 product of the subtraction of chick fibroblast poly(A) cDNA (driver) from day 9 calvarial poly(A) cDNA (tracer). Methods for the isolation of positive cDNA clones, preparation of plasmid DNA and sequence analysis were according to the protocols provided by Stratagene.

DNA Sequence Homology Searches

Sequences were used in FastA DNA homology searches against the Genbank and EMBL DNA sequence databases [Pearson and Lipman, 1988].

RESULTS

Characterization of Chick Calvarial Cells in Culture

To confirm the osteogenic phenotype of the calvarial cells in culture, the cultures were tested for the presence of alkaline phosphatase and calcium mineral (Fig. 1). Chick fibroblasts grown for 15 days under the same conditions were used as control cultures for the stains. No calcification of calvarial cultures was seen prior to the cells reaching confluence. By day 9, small areas of mineral deposits could be seen at the microscopic level (Fig. 1) and by day 15, cultures contained large foci of calcification which could be seen both at the macroscopic and microscopic level (Fig. 1). Day 9 calvarial cells showed intense staining for alkaline phosphatase activity whereas staining at day 15 was less intense. This was consistent with findings by Gerstenfeld et al. in that their β-glycerophosphate-treated cultures showed a decrease in alkaline phosphatase activity with time in culture. Chick fibroblasts cultured under the same conditions stained negative for alkaline phosphatase and calcium mineral.

Production of a cDNA Probe Specific to Mineralizing Chick Calvarial Cells

The subtractive hybridization technique [Brady and Iscove, 1993; Brady et al., 1995] was used to identify genes expressed by mineralizing chick calvarial cells in culture but not by chick fibroblasts in culture. Subtractive hybridization was performed using as a template, RNA extracted from chick calvarial cells in culture at both day 9 and day 15 (tracer) and chick fibroblast RNA extracted from cells cultured under the same conditions as the chick calvarial cells (driver). The two time points in the calvarial secondary culture were chosen since day 9 coincided with the early stages of mineralization, whereas by day 15 large foci of calcification were observed. In both instances, four rounds of subtraction were performed. For both sets of subtractions, equal quantities (~100 ng) of the poly(A) PCR products for the driver, tracer and reamplified subtracted material (S1–S4) were separated on 1.4% agarose gels, and blotted onto nitrocellulose. Two blots for each subtraction were prepared. One of the day 9 blots (Fig. 2A) and one of the day 15 (Fig. 2B) blots was hybridized with the S4 product of the day 9 and day 15 subtractions, respectively. A gradient of hybridization of the probe was seen, with least hybridization to the driver material,

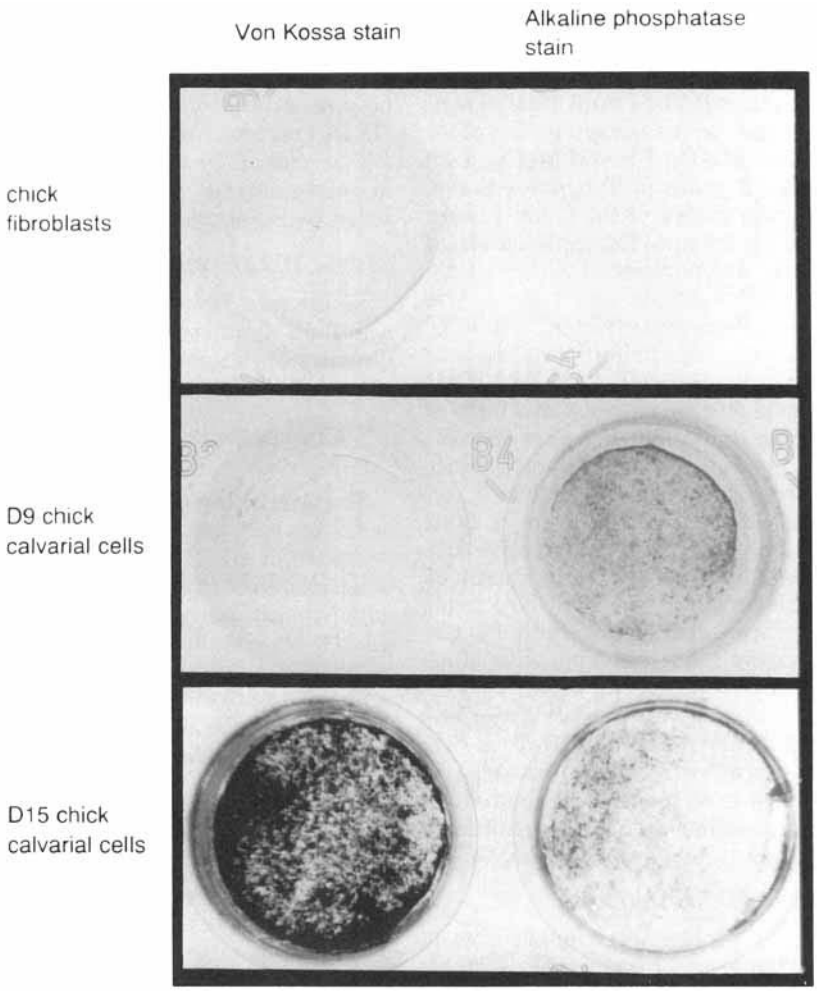

Fig. 1. Cultures grown in 24-well dishes (well area ~1.8 cm^2) were fixed with 2% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4, for 2 min, rinsed with fresh buffer, and stained for mineral using the von Kossa method [Clark, 1981] (left hand wells). Cultures fixed and rinsed in the same way were stained for alkaline phosphatase using the Sigma Fast™ kit (right hand wells). The middle and bottom panels show stains on chick calvarial cells at day 9 and day 15 of secondary culture, respectively. Second passage calvarial cells had been grown in 10% fetal calf serum in BGJ_b (Fitton-Jackson modification) supplemented with ascorbate (50 μg/ml) and the alkaline phosphatase substrate, β-glycerophosphate (10 mM final concentration).

and most to the final subtraction. This gradient of hybridization was more pronounced for subtractions between day 9 calvarial cells and fibroblasts than between day 15 calvarial cells and fibroblasts. The observed hybridization pattern was consistent with removal of common sequences between the driver and tracer and consequently an enrichment of tracer-specific sequences. Conversely, when driver material was used as a probe, the reverse pattern of hybridization was seen, with a decrease in hybridization as the subtractions proceeded (results not shown). This reduction in hybridization with subtractions again demonstrated the removal of common sequences between the driver and tracer cDNAs.

The second set of blots was hybridized with the 350 base pair 3′ end of the COL1A1 cDNA (Fig. 3A for the day 9 subtraction and Fig. 3B for the day 15 subtraction) as a further means of testing the efficiency of the subtraction procedure. Since both calvarial cells and fibroblasts express COL1A1, this cDNA should be removed from the subtracted products. When hybridized to the Southern blot of driver, tracer, and reamplified subtractates, it was seen that both fibroblasts and day 9 and day 15 calvarial cells expressed COL1A1 (Fig. 3A,B). In the subtractions between day 9 calvarial cells and fibroblasts, hybridization of the COL1A1 probe was reduced dramatically in the final subtraction. However, cDNAs for COL1A1 do not appear to be removed effectively in the subtractions between day 15 calvarial cells and fibroblasts.

Identification of Mineralization-Specific Genes by cDNA Library Screening

The Southern blotting results indicated that the subtractions between day 9 calvarial cells and fibroblasts showed a better enrichment for tracer-specific sequences

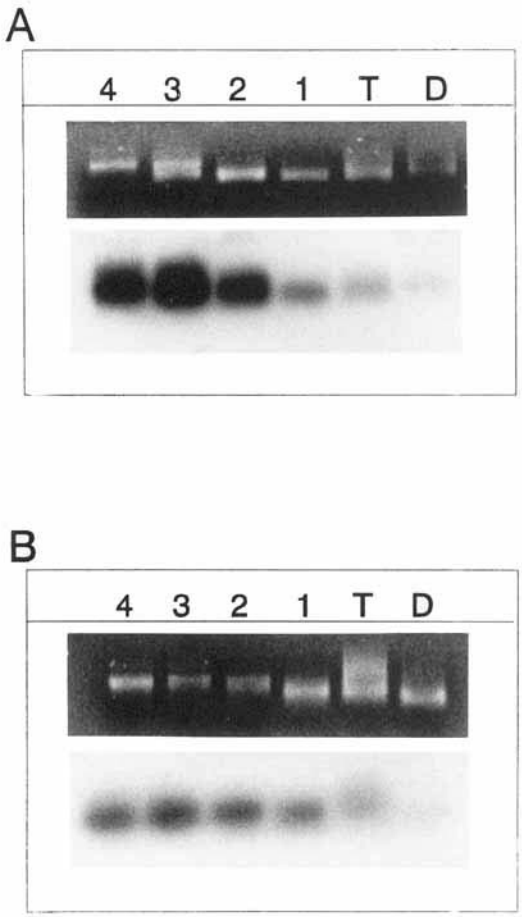

Fig. 2. Approximately 100 ng of driver, tracer and reamplified cDNAs were electrophoresed for a limited time through 1.4% agarose and blotted onto nitrocellulose. The blots were hybridized to ^{32}P-labeled probes prepared from the S4 cDNA pool and exposed to X-OMAT AR film. D, driver cDNAs prepared from chick fibroblasts; T, tracer cDNA prepared from either day 9 or day 15 second passage calvarial cells; 1, 2, 3, and 4, cDNA obtained from sequential rounds of subtraction. **A:** Top panel, ethidium bromide stained agarose gel of cDNAs obtained from subtractions between day 9 calvarial cells and fibroblasts. Bottom panel, Southern blot of gel hybridized with the S4 cDNA pool. **B:** Top panel, ethidium bromide stained agarose gel of cDNAs obtained from subtractions between day 15 calvarial cells and fibroblasts. Bottom panel, Southern blot of gel hybridized with the S4 cDNA pool.

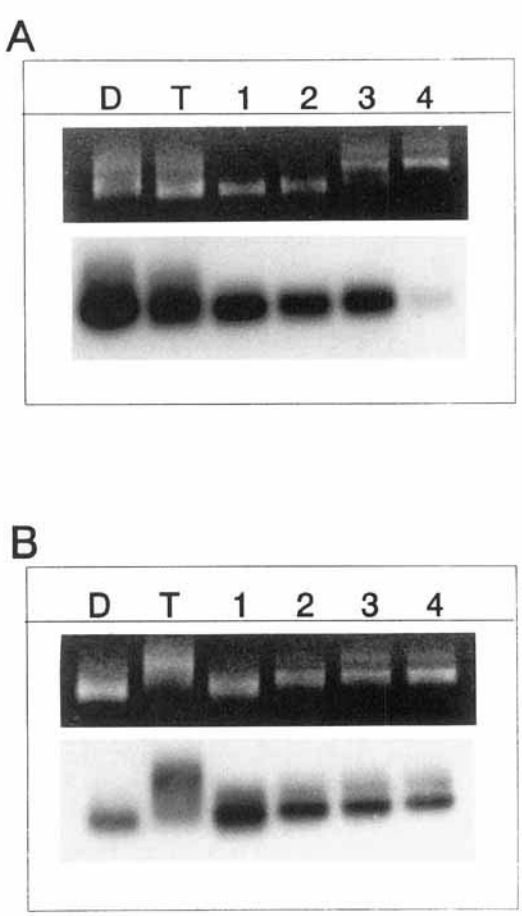

Fig. 3. Approximately 100 ng of driver, tracer, and reamplified cDNAs were electrophoresed for a limited time through 1.4% agarose and blotted onto nitrocellulose. The blots were hybridized to ^{32}P-labeled probes prepared from the 3′ end of COL1A1 cDNA and were exposed to Kodak X-OMAT AR film. D, driver cDNAs prepared from chick fibroblasts; T, tracer cDNA prepared from either day 9 or day 15 second passage calvarial cells; 1, 2, 3, and 4, cDNA obtained from sequential rounds of subtraction. **A:** Top panel, ethidium bromide stained agarose gel of cDNAs obtained from subtractions between day 9 calvarial cells and fibroblasts. Bottom panel, Southern blot of gel hybridized with the COL1A1 cDNA. **B:** Top panel, ethidium bromide stained agarose gel of cDNAs obtained from subtractions between day 15 calvarial cells and fibroblasts. Bottom panel, Southern blot of gel hybridized with the COL1A1 cDNA.

than between day 15 calvarial cells and fibroblasts. We therefore decided to screen the chick calvarial cDNA library with the S4 cDNA pool from day 9 calvarial cells. Approximately 100,000 pfu were screened using the ^{32}P(dCTP) labeled S4 probe. Ten positive clones were identified on duplicate filter lifts. These clones were used in secondary screening to obtain individual isolated positive plaques. For each of the ten isolated clones, the inserts were excised from the lambda ZAP phage vector in pBK-CMV phagemids. Excision of the pBK-CMV phagemid was achieved by co-infection with lambda ZAP phage from isolated secondary clones and ExAssist™ filamentous helper phage (M13) [Stratagene; Short et al., 1988; Short and Sorge, 1992]. The excised pBK-CMV phagemids from each of the ten clones were purified, and the presence and size of each of the cDNA inserts determined. Insert sizes ranged from approximately 800 base pairs to approximately 6 kilobases. The ten cDNA clones isolated were grouped by cross hybridization to determine which of the clones represented overlapping cDNAs or unique cDNA species. Three groups of unique sequences, groups A to C, were found consisting of 8, 1, and 1 clones, respectively.

Identification of the cDNA Groups by Sequencing Analysis

One member of each of the three groups of clones was sequenced from purified phagemid vectors using the T3 and T7 primers which flank the cDNA insert (Fig. 4). The sequence from group A (clone 1) from the T3 primer showed 86.2% identity in 109 overlapping base pairs with the 3′ end of the chick osteopontin (also called

Sequence from clone 1 revealed 93.2% identity in 88 bp overlap with the 5' end of osteopontin cDNA from *Gallus Domesticus*

```
                10        20        30        40        50        60
clone1 GAAAGCCAGAGCGTCACTCAGCCCGCAGTAGGAGTTGCTGCTGGGATTGCCGGAG-CCCTTTGAGCAG
       |||||||||||| ||||||||||||||||||||||||||||||||||||||||| ||||||||||||
chkost GAAAGCCAGAGCCTCACTCAGCCCGCAGTAGGAGTTGCTGCTGGGATTGCCGGAGCCCCTTTGAGCAG
        1220      1230      1240      1250      1260      1270      1280
490

       70        80        90
clone1 CTGACT--TTCAGCACAGAAGTAA
       ||||||  | ||||||||  |
cc18sr CTGACTTCTCCAGCACAGGAAAGG
          1290      1300
```

Sequence from clone 10 revealed 97.3% identity in 113 bp overlap with cystatin cDNA from *Gallus Domesticus*

```
         109       99        89        79        69        59        49
clone10TATATTTGGACTATCTGTTTGTCTTCTCGCAGCTTTCCCAATAAAGCAACTCCAGTGTCAGAATTGAA
       ||| |||||| || ||||||||||||||||||||||||||||||||||||||||||||||||||||||
chkcys TATTTTTGGAGTAGCTGTTTGTCTTCTCGCAGCTTTCCCAATAAAGCAACTCCAGTGTCAGAATTGAA
       5460      5470      5480      5490      5500      5510      5520

            39        29        19        9
clone2 GATGAAGTTGGTGTCCCTATGAAGTGACTACTGTAAACATTAAAA
       |||||||||||||||||||||||||||||||||||||||||||||
chkcys GATGAAGTTGGTGTCCCTATGAAGTGACTACTGTAAACATTAAAA
         5530      5540      5550      5560      5570
```

Sequence from clone 3 revealed 96.9% identity in 65p overlap with bone sialoprotein-II cDNA from *Gallus Domesticus*

```
         159       149       139       129       119       109
clone3 ACTACGGCTACTACAAGGGGCATGGCTATGACATGTATGGGCAGGAATACTACTACAACCAGTGA
       | ||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
chkBSP AGTACGGCTACTACAAGGGGCATGGCTATGACATGTATGGGCAGGATTACTACTACAACCAGTGA
         770       780       790       800       810       820       830
```

Fig. 4. Sequences derived from the mineralization-specific cDNA clones were used in DNA sequence database (EMBL and GenBank) homology searches using FastA. The sequence identities were close to 100%. The sequence differences may have arisen from PCR and DNA sequencing errors or represent natural sequence variations between different breeds of chicken.

bone sialoprotein I or BSP-I) cDNA sequence. Sequence obtained using the T7 primer demonstrated 93.2% identity in 88 base pairs of overlapping sequence with the 5′ end of the chick osteopontin cDNA sequence. Sequence from group B (clone 10) from the T7 primer had 97.3% identity in 113 overlapping base pairs with chick cystatin cDNA. Sequencing of group C (clone 3) from the T3 primer showed 96.9% identity in 65 base pairs of overlapping sequence with the cDNA sequence for bone sialoprotein II.

DISCUSSION

The aim of the experiments described was to develop a system with which to identify genes involved in bone formation and the mineralization of collagen. The procedure used to achieve this was a PCR-based subtractive hybridization technique to generate a heterogeneous cDNA probe which was enriched for genes expressed by mineralizing cells. Two sets of subtractions were performed. In these experiments, PCR prod-

ucts generated by RT-PCR of mRNA from chick fibroblasts were subtracted from those generated from calvarial cells grown to day 9 of secondary culture and separately from calvarial cells grown to day 15 of secondary culture. Day 9 cells were chosen as they represent an early stage of mineralization, whereas, by day 15, mineralization was well advanced. Southern blotting indicated that the subtractions between day 9 calvaria cells and fibroblasts showed a greater enrichment for calvarial-specific genes than the subtractions between day 15 calvaria cells and fibroblasts. This was most obvious when both sets of subtractions were hybridized with a probe for COL1A1. COL1A1 cDNAs, which were common to both calvarial cells and fibroblasts, were effectively removed in the day 9 calvaria-fibroblast subtraction. In contrast, the COL1A1 gene was not removed in the day 15 calvaria-fibroblast subtraction. One explanation for this might be that calvarial cells at day 15 of secondary culture may upregulate COL1A1 expression in response to mineralization. Another explanation might be that in the day 15 calvarial cells, early onset genes (e.g., osteopontin) may be down-regulated.

Having established from the Southern blots that the subtractions between day 9 calvarial cells and fibroblasts showed removal of common sequences and consequently the enrichment of calvarial-specific genes, the S4 cDNA pool was used to screen a chick calvarial cDNA library. The library was prepared from a mixture of chick calvarial cells in culture and calvaria tissue. Three groups of cDNAs were identified. These cDNAs represented the genes encoding osteopontin, bone sialoprotein II, and cystatin. The genes encoding these proteins are known to be expressed during mineralization providing a positive indication that there had been an enrichment of mineralization-specific genes in the subtractions. Osteopontin is a sialoprotein found in mineralizing bone [Mark et al., 1987] and dentin [Mark et al., 1988]. The amino acid sequence deduced from a cDNA clone shows the presence of a GRGDS cell attachment sequence and a stretch of nine aspartic acid residues which may confer hydroxyapatite binding capacity [Oldberg et al., 1986]. It has been postulated therefore to play a role in the attachment of cells to the mineralized matrix. Bone sialoprotein II (BSP-II) is produced by osteoblasts and osteocytes [Bianco et al., 1991] and contains the RGD cell attachment sequence [Oldberg et al., 1988]. BSP-II has been shown to have a significant affinity for collagen at physiological ionic strength [Fujisawa and Kuboki, 1992] suggesting again that BSP-II, like osteopontin, mediates attachment of osteoblasts to collagen in the bone matrix. BSP-II has also been implicated in initiating mineralization by nucleating hydroxyapatite formation [Hunter et al., 1993]. Cystatins are inhibitors of thiol [Isemura et al., 1986] proteases and thiol protease activities are known to be involved in bone turnover [Goto et al., 1993; Delaissé et al., 1980]. Cystatin may therefore have been upregulated in the mineralizing calvarial cells in culture to facilitate the deposition of a mineralized matrix by inhibiting proteases causing matrix degradation.

From these results, it was concluded that the product of the final subtraction between day 9 calvarial cells and fibroblasts provides a useful probe with which to screen our entire cDNA library. As only 1/42 of the library was screened in this preliminary study, there is a potential to identify most of the genes involved in the mineralization of calvaria, some of which may be novel.

ACKNOWLEDGMENTS

The work is funded by Action Research (S/P/2355) and the Wellcome Trust (19512). KEK is a recipient of a Senior Research Fellowship in Basic Biomedical Science from the Wellcome Trust.

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